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STUDIES ON OXIDATION-REDUCTION

XII. A NOTE ON THE SCHARDINGER REACTION (IN REPLY TO KODAMA)

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STUDIES ON OXIDATION-REDUCTION

XII. A NOTE ON THE SCHARDINGER REACTION

(In reply to Kodama)

In an article on methylene blue, Clark, Cohen, and Gibbs (1925) introduced a brief note describing time: potential curves observed with milk treated with formaldehyde in the absence of methylene blue. Kodama (1926) states in a later paper that he was "entirely unable" to repeat our results. He also adds the footnote: "Dr. M. Dixon has been unable to repeat Clark's [1] results." Kodama uses our data on bacterial cultures as a means of casting doubt upon our interpretation of the potential changes. We concluded that the milk-formaldehyde experiments had to do with the Schardinger reaction, but Kodama says: "Occasionally, however, curves similar to Clark's have been obtained when a not very fresh milk was used, but these potential changes were obviously due to bacteria, and were not dependent on the presence of aldehyde. It is significant that the final potential reading in these experiments, which was the same as that obtained by Clark, after inoculating the milk with bacteria, was identical with the final value given by Clark for the Schardinger system; this fact suggests that the effects he observed may possibly have been due to bacteria."

We would have relieved Kodama from making this statement if we had elaborated our brief note and had published controls. Instead of now discussing what appeared to us adequate controls in the older experiments, we shall give briefly the results of a repetition of the work.

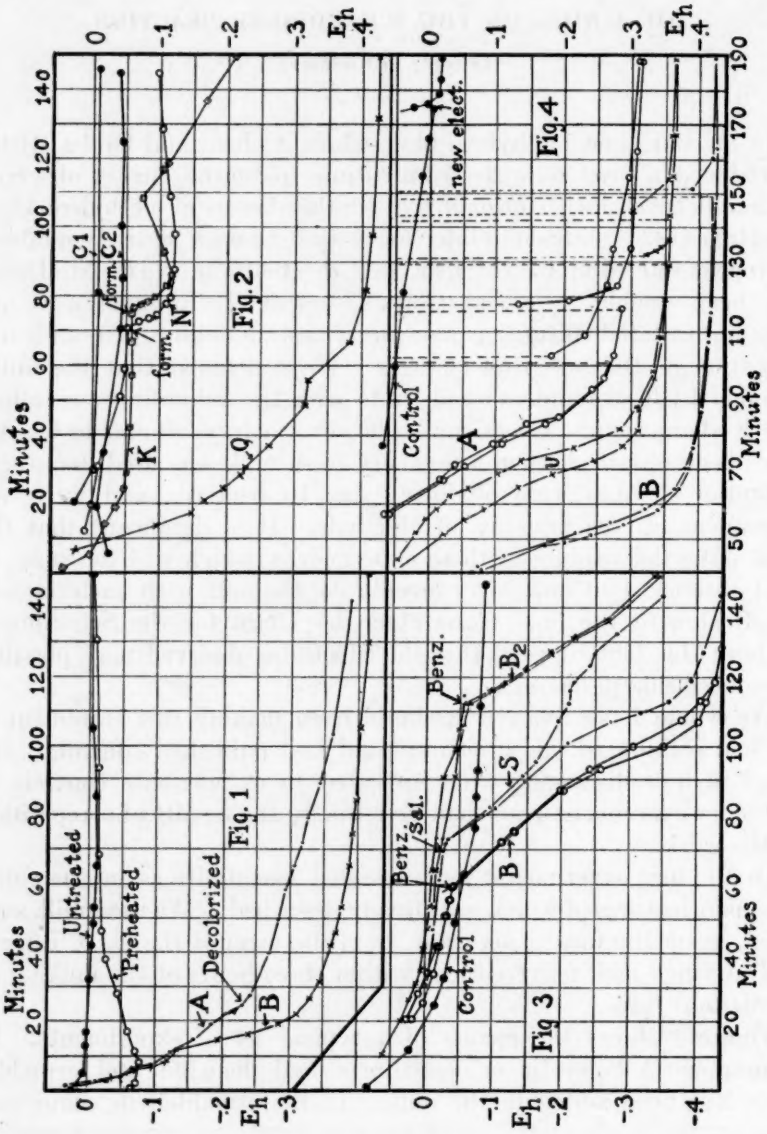
In the first experiments we proceeded essentially as in the older, the main features of which are already described. We used milk supplied, as on the former occasion, from the farm of the U. S. Bureau of Dairying [2] and delivered iced within three hours of the milking at the distant farm.

Figure 1 shows the results of a typical set of experiments. In Experiment A, Schardinger's mixture of methylene blue and formaldehyde had been added to the milk. In B, formaldehyde alone had

[1] Kodama here refers to the paper by Clark, Cohen, and Gibbs (1925).

[2] We take this opportunity to express our appreciation of the aid rendered by Doctor Rogers and Mr. Woodward. They cooperated in supplying promptly, after a special and cleanly conducted milking, the samples used in our experiments. We thank Mr. Hall of this laboratory for the use of his automobile in making the last lap of the journey. Cooperation at all points seemed to have been responsible for the fair uniformity of our controls.

been added. Distinct drops in potential are evident, and these contrast with the fairly uniform level of potential in the untreated control and with the course of potential change in the heated milk. We repeated this experiment several times and on one or two occasions



included a control with buffer and aldehyde alone. The results were essentially the same and fully confirm the essential feature of our previous work.

Inasmuch as a possible *ultimate* bacterial origin of the Schardinger "enzyme" has been one of the chief topics of the literature on the

subject, it may perhaps be well to emphasize the fact that these and the following experiments are not concerned with this problem. The only concern is with an adequately clear distinction between effects obviously accelerated by the addition of an aldehyde and those which might be ascribed to the *multiplication* of bacteria. For this purpose we have considered adequate the comparisons between effects obtained in the presence and in the absence of an aldehyde. When a very distinct acceleration of potential change follows the addition of the aldehyde, when no comparable potential change occurs in the untreated milk, and when no reduction of methylene blue occurs in the untreated milk, we assume that we have met elementary requirements comparable with those which originally led to the designation of a differential effect as "the Schardinger reaction." Since we used the methylene blue reduction test (in absence of aldehyde) and potential changes in the absence of aldehyde as rough indications of bacterial action, it is appropriate to state that we did not aim at sterile conditions; but we depended upon the conclusions of Hastings, Davenport, and Wright (1922) that the glassware need not be sterile, but clean for the methylene blue reduction test. We used strong alkali and strong bichromate followed by rinsing with freshly distilled water [3] in cleaning all glassware and did not guard against air contamination. Our experience has been that if vigorous reduction by bacterial growth is to be expected within the duration of our experiments, the culture picked up must already have reached its period of logarithmic growth or must be a heavy inoculation. Indeed, we inoculated one control with a drop of one-day-old sour milk without noting within the ordinary period of our experiments the fall of potential or decoloration of methylene blue which a culture, taken in its period of logarithmic growth, would be expected to give after the first few hours.

With regard to the milk itself, we have the assurance of the Bureau of Dairying that it was drawn with particular care as to cleanliness. In only two out of a large number of cases was there a bare suggestion that the control experiments were vitiated by bacterial growth. Lacking bacterial counts in these cases, we perhaps should not accept the exceptionally steep time: potential curves as evidence of bacterial growth. However, we have so done and have arbitrarily ruled these two cases out of consideration.

Although Kodama's main experiments were at low temperature and with acetaldehyde, he states that he covered the conditions of higher temperature and the use of formaldehyde. Failure to confirm our results is, therefore, not to be sought in these special conditions of his main experiments.

[3] We did not examine this water. It was used for cleaning and for preparation of aldehyde solutions.

The radical departure from customary procedures was Kodama's use of a stream of nitrogen "which has passed through two wash bottles containing strong pyrogallol solution."

Now, it so happened that the first experiment which we made with a stream of nitrogen duplicated Kodama's results. It was a very crude preliminary experiment, and the only peculiar conditions we noted were, first, the use of a tube of copper gauze which had become almost completely oxidized during the passage (through the furnace) of nitrogen containing 1.87 per cent O_2; second, the use of spiral wire electrodes which were found to have trapped the butter formed during the agitation of the milk by the nitrogen stream. Numerous repetitions of the experiment with plate electrodes and deoxygenated nitrogen failed in every way to yield the first result.

The nearest approach was in an experiment (fig. 2, curve K) under the following conditions: A special vessel held 10 c. c. milk (Kodama used 5 c. c.; our ordinary vessels required 50 c. c.). The milk was led through a short piece of capillary tubing to a wider tube of milk, making liquid junction with the calomel half-cell. Nitrogen was led in through another capillary small enough to produce slight back pressure and thus maintain a steady stream through the milk as the gas came bubble by bubble through two ordinary wash bottles containing about 400 c. c. of freshly prepared Hempel's pyrogallol solution. The electrode was gold-plated platinum foil, the temperature 30° C., the milk about three hours old. The nitrogen from a tank was known to contain 1.87 per cent of oxygen before entrance to the pyrogallol solution. Connections between absorption bottles and electrode vessel were made with fresh *rubber tubing.*

The points indicated by the centers of squares in Figure 2 outline the course of the time : potential curve. With the scale used, there do not appear the slight but definite changes in direction of potential drift occasioned by alteration of the velocity of gas flow; but, since these changes of the direction of potential drift might have been due to effects other than that expected from a slight residual oxygen content, no importance is attached to the numerical details. The fall of potential on addition of formaldehyde was definite. Its recovery was similar to that observed when the raw, untreated nitrogen was used. An example of results obtained with untreated nitrogen is shown by curve N, Figure 2. For comparison there is given the time: potential curve observed with the same milk in a quiescent tube not deaerated and under treatment with formaldehyde alone, curve Q. The two controls of untreated quiescent milk are shown by C_1 and C_2.

Now, we have never been under the delusion that the water-pumped, Linde process nitrogen we have been using is *absolutely* free from its residual oxygen after passage through a large tube of

reduced copper gauze heated in a furnace to about 730° C. Yet, when so treated and led through glass and copper tubing with no rubber connection,[4] the nitrogen has been satisfactory for our studies of dilute solutions of reversible oxidation-reduction systems.

When nitrogen so treated was used, we only occasionally observed the approach of the untreated milk to constant potential. The curves of the controls in Figures 3 and 4 are somewhat flatter than those often observed, but they are fairly representative of the usual course of the control and show distinct drifts to more negative potentials. In two instances *very* much more rapid drifts were observed, and these were the arbitrarily rejected cases mentioned above as suspected cases of high bacterial activity.

If, to a deaerated milk, running such a course as illustrated in Figure 3, we added formaldehyde, salicylaldehyde, or benzaldehyde, we observed a decided acceleration in the potential change.

In two of the cases illustrated (B_1 and B_2) there was added to 50 c. c. milk 1 c. c. of a solution made by dissolving 1 c. c. of benzaldehyde in 10 c. c. ethanol. Each pair of curves illustrates the conduct of two gold-plated, platinum electrodes in the same sample of milk. In a third case of this same series of experiments there was observed a divergence of the potentials of the two electrodes similar to that illustrated in Figure 3 by the S set of curves. This particular set, S, was obtained with addition of salicylaldehyde. Two other parallel experiments with salicylaldehyde yielded closely agreeing results comparable to those of the two benzaldehyde cases illustrated. We have introduced the discrepant pair of curves for the salicylaldehyde case in place of the similar set of curves for the benzaldehyde case, because in the former case we replaced the more erratic electrode and after six minutes found the two electrode potentials to be identical. The discrepant results shown give a fairer picture of what often happens. The introduction into one chart of results with benzaldehyde and with salicylaldehyde saves space.

In one experiment, milk treated with salicylaldehyde was held in one case at 30° and in the other case at 40° C. The time : potential curves for duplicate electrodes in each case separately ran close to one another. In the 30° case the potential[5] dropped from +0.16 to −0.12 in 30 minutes. In the 40° case the potential dropped from +0.16 to −0.12 in five minutes.

As is well known, the heating of milk near its boiling point in the absence of oxygen results in a reducing action, as evidenced by the reduction of various dyes. A milk heated in the absence of any dye to 90°–95° C., maintained within this range for 10 minutes while a stream of nitrogen passed and then cooled to 42°, gave with one elec-

[4] Junction of glass and copper was made with de Khotinsky cement.
[5] All potentials in this paper are referred to the hydrogen standard.

trode a potential of −0.106 and the other −0.125. The drift of potential was then very slowly becoming more positive. Addition of benzaldehyde caused first a considerable jump of the order of 0.1 volt in the *positive* direction. It was followed by a fairly stable potential near −0.06 for one electrode and −0.08 for the other. We have not pursued the subject further; but aside from the interesting effect of heating, this serves as a partial additional control.

Usually the potential-changes after addition of benzaldehyde or salicylaldehyde were somewhat more extensive than after addition of formaldehyde. With deaerated milk to which formaldehyde was added, there was often obtained a flattening of the curve near −0.2 volt.

Attempts to discover, by the potentiometric method, the effects of initial oxygen content, temperature coefficients, etc., were not successful. The individual electrodes were too erratic to yield those reproducible *quantitative* data which would carry conviction were we to deal with the distinct hint that the effects of oxygen content, temperature, etc., are detectable by the electrode method.

Our experience has led us to suspect that among the various reactions in which an aldehyde can take part when added to milk, the combination with protein is particularly significant in the case of formaldehyde in that slightly different conditions may yield distinctly different results. We suspect, but have not proved, complications due to loss of aldehyde, uneven distribution of aldehyde between plasma and fat (especially the butter formed during deaeration with a stream of nitrogen), destruction of "enzyme," and numerous minor matters.

Since we are not dealing with equilibrium potentials, we can not agree to use Kodama's expression "the true reducing potential of the enzyme system." Furthermore, it is impossible to apply the criteria of significance applicable to systems in equilibrium. This was discussed by Cannan, Cohen, and Clark (1926). However, we shall presently note a phenomenon of possible significance.

Perhaps the difficulty in obtaining reproducible results is due in large measure to the peculiarities of individual electrodes; and because of this we are less confident than we were of the ability of the method to reveal with certainty the detail of pH effects or to yield data of the accuracy and reproducibility required for certain kinetic studies.

In Figure 4 are charted the later sections of curves showing the course of experiments designed to reveal the pH effect. Curves A (acidified) are for 50 c. c. milk acidified with 5 c. c. N/5 HCl and treated with 0.2 c. c. alcoholic benzaldehyde. Curves U (untreated) are for 50 c. c. milk +0.2 c. c. alcoholic benzaldehyde. Curves B (base treated) are for 50 c. c. milk treated with a base, namely, 5 c. c. N/5 NaOH and then with 0.2 c. c. alcoholic benzaldehyde.

In each case the benzaldehyde solution contained 1 c. c. benzaldehyde in 10 c. c. ethanol. For the sake of uniformity of scales and to save space, only the latter parts of the curves for this experiment are shown in Figure 4.

When no very decided differences in the *slopes* of the time: potential curves were noted, it was realized that hydrogen electrode measurements of the milk before and after treatment with the acid and the alkali would add information of little value, and attention was turned to the stability of the potentials observed. At points indicated by the origin of vertical dotted lines, one or the other of the pair of electrodes was short-circuited with the calomel half-cell by connecting the mercury leads with freshly scraped copper wire (B. S. gauge 18). Since the calomel half-cell was +0.244 volt and was probably very much less easily polarized than the small, bare, gold electrode, the potential difference at the gold electrode must have approached +0.244 on the scale of the figure during the 10 to 20 seconds of short-circuit. The recoveries are indicated in the figure and seem to us evidence of a rather surprising stability. Other experiments to test the stability of the potentials were performed in other cases. For instance, while two electrodes in formalized milk, deaerated with nitrogen, were giving closely agreeing time: potential curves, one electrode was withdrawn, replated, or polarized in various manners. In one case of drastic anodic polarization of a gold-plated electrode, a very positive potential which did not drift for a considerable time was observed. But in other cases a new or treated electrode would quickly assume a value very near that of its mate, as was the case in the control of Figure 4. If there was delay, the potential drift was in the direction of recovery of the original value. However, the rates of this recovery varied greatly.

Now, Cannan, Cohen, and Clark (1926) have discussed the very slight poising action of biological systems and have emphasized the consequent difficulties in the interpretation of potential changes. Indeed, it was the realization of this in experiments conducted by Clark in 1919 that led to the development of oxidation-reduction indicators (Clark, 1920) with which the principles concerned could be better studied. When, therefore, an electrode is introduced into so complex a heterogeneous system as whole, fresh milk, with its obvious inequalities of fat distribution, air bubbles, dispersed particles, etc.; when, in addition, it is supposed to follow a reaction which is not only rapid but involves the distribution of reagent between fat and aqueous phases, protein combination and "enzyme" transformation, oxygen clean-up, etc., there is hardly to be expected uniform responses. The remarkable thing is that any order can be observed. The situation is different when methylene blue is present. Its system is known to "take hold" of the electrode, as it were; and

the fact that potential changes are then accelerated may possibly, although by no means certainly, have no other significance than an accelerated depolarization so far as a comparison of electrode responses is concerned. Indeed, by properly adjusting conditions so that a very rapid decoloration of methylene blue is observed (e. g., 5 minutes or less), decoloration often can be observed before the potential has caught up to the value predicted from the known potentials of the methylene blue system in homogeneous solution. The discrepancies are often wide enough to allow for all reasonable protein and "salt" effects, pH changes, etc., and clearly indicate that instantaneous response is not obtained even with a definite electromotively active system when an electrode is smeared with the material it inevitably accumulates in milk.

However, with the exception of one crudely performed preliminary experiment in over fifty, we did not fail to obtain a well accentuated acceleration of potential change on addition of an aldehyde alone.

THE XANTHINE OXIDASE-HYPOXANTHINE SYSTEM

The situation is remarkably different with mixtures of "xanthine oxidase" and hypoxanthine. Here we are pleased to confirm Kodama's observations.

The xanthine oxidase was prepared by the method of Dixon and Kodama (1926). Aside from treatment by ether to remove fat, there was no further purification of the enzyme precipitated from whey by half saturation with ammonium sulphate. The hypoxanthine was an old preparation, for which we are indebted to the Division of Soil Fertility, Bureau of Plant Industry, United States Department of Agriculture.

As Kodama showed, and we confirm, mixtures of buffer, "xanthine oxidase," and hypoxanthine cause no distinct drift of electrode potential; but when an indophenol or methylene blue is added, the potential promptly runs through the zone characteristic of the indicator used. The remarkable aspect of the matter, and one which should not escape notice, is that the potential indicates extreme reduction of the dye. In one case the potential, originally in the region of only slightly reduced methylene blue, dropped in two hours to -0.116 volt at pH 7.6. Since methylene blue in homogeneous solutions is half reduced at -0.008 volt (pH 7.6), it is readily calculated that, were the solution homogeneous, the methylene blue should have been reduced to the extent of about 99.97 per cent. In another case of more rapid reduction the potential was followed long enough (1.5 hours) to indicate the order of 99.9999 per cent reduction, and slow drift toward more negative potentials had not yet ceased. Needless to say the potentials of controls showed no significant drift. Kodama attributes a flattening of his time : potential curves to

residual oxygen. For the extreme potentials, thorough deaeration seems essential; and since methylene white may aid in the oxygen clean-up, the way may thus be cleared for the slight activity of cellular material. This may explain the extreme drift of potential. If it does not account for the drift and "xanthine oxidase" be regarded as a catalyst, interesting questions arise.

Excluding the extreme potentials as artefacts, so far as the main problem is concerned, we have in the reaction of the systen—"xanthine oxidase"-hypoxanthine-methylene blue—a striking phenomenon. The absence of electromotive activity when the dye is not present seems to have been the origin of Kodama's remark—

"* * * the * * * characteristic feature of biological oxidation-reduction systems [is] that, while giving no reduction potentials themselves, they are yet able to bring about reduction of a large number of hydrogen acceptors."

Would it be unfair to cite, as a refutation of the *generality* of this statement, our data on bacterial cultures which Kodama uses as a means of throwing doubt on the significance of our work with the Schardinger reaction?

We are quite ready to accept the conclusion that, except as a means of studying the course of dye reduction, the electrode may prove to be useless in studying the "xanthine-oxidase." We see nothing unique in this, since the electrode has repeatedly proved useless in the study of very many inorganic and organic oxidation-reduction processes; and there seems to be no way of definitely predicting *a priori* what the limitations of the electrode method are.

In summary we may say that while we confirm Kodama's observations with the "xanthine oxidase"-hypoxanthine system, we can not explain his failure to confirm our observations with the Schardinger reaction. It might be inferred, from our remarks on the effects of "deaeration" with impure nitrogen, that we suspect Kodama's results to have been due to the use of pyrogallol which is well known to be a slow absorbent. However, we wish it clearly and unequivocally to be understood that we can not imagine a balance between oxidative and reductive tendencies to have been so nicely adjusted by accident as to have misled Kodama and also (according to Kodama) Dixon. We are aware of the fact that other phases of the Schardinger reaction have failed of duplication in different laboratories. We, therefore, do not explain Kodama's results; we report our own.

Furthermore, since there is now evident a problem in the duplication of experimental fact, further speculation had best await clarification of fact by a third laboratory, for nothing is quite so fatal to progress as a contentment with preconceptions which dulls interest in verification and in agreement as to fact.

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